anion-exchange chromatography to be orthophosphate (14 %), acid-stable phosphate (3 %), pyrophosphate (23 %), tripolyphosphate (44 %), and a fraction retained by the resin (16 %). This last fraction probably consists of more highly condensed polyphosphates.

3. The composition of the water-soluble phosphates excreted by *A. grisella* was orthophosphate (29 %), pyrophosphate (23 %), tripolyphosphate (40 %), acid-stable phosphate (2 %), and the fraction retained by the resin (6 %).

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A Method for the Determination of Non-Haem Iron in Bone Marrow

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There are many reports in the literature (Rath & Finch, 1948; Masshoff & Gruner, 1951; Hutchison, 1953) of investigations into the iron content of bone marrow from human subjects by various histological techniques. Most of the work has been done with smear preparations or sections of fixed tissue from material obtained at biopsy and stained with acidified potassium ferrocyanide. A deposit of ferri-ferrocyanide (Prussian blue) indicates the presence of iron-containing material. It is well recognized that only haemosiderin will be detected in this way, the Prussian blue being formed on the surface of the haemosiderin particles.

Granick (1954) originally postulated that iron was utilized in the synthesis of haemoglobin only from ferritin and that haemosiderin represented deposits of iron in excess of metabolic requirements. In these circumstances the presence of haemosiderin would imply the presence of adequate amounts of iron and the Prussian blue test could be used as a rough estimate of the body stores of iron. Shoden, Gabrio & Finch (1953), however, have found a simultaneous labelling of both ferritin and haemosiderin after administration of radioactive iron. Similarly, during haemopoiesis they found that iron from both ferritin and haemosiderin was utilized. These findings would suggest the necessity of estimating the total quantity of iron in the bone marrow rather than that fraction present as haemosiderin.

Cavayé Hazen (1949) and others have reported the results of the estimation of iron in bone marrow obtained at necropsy in a variety of diseases. Relatively large amounts of tissue were required for the analysis which was performed on a wet ash of the material.

As it was desirable to use biopsy material it was decided to develop a method requiring only a small quantity of marrow tissue. It has been shown by Krause (1943) and by McCoy & Schultze (1944) that the lipid content of marrow tissue is variable. Dietz (1949) has studied the relationship between nitrogen, lipid and water content in bone marrow from several species. From these results it would appear that any analysis based on tissue weight, either wet or dry, would be unreliable. Davidson, Leslie & White (1947) have stated that with moderate quantities of marrow tissue (30–60 mg.) the values for deoxyribonucleic acid are the least satisfactory of their results, the amount of phosphorus being too small for accurate analysis. It was therefore decided to try to relate the iron content of the marrow to the protein content and this has proved satisfactory. The method for the

measurement of protein is relatively specific, is simple to use and was designed especially for the estimation of small amounts of protein.

A description of the method is given, together with a comparison of the results obtained by this method and by the classical Prussian-blue staining technique.

The results of the measurement of the iron content of bone marrow obtained from a group of hospital patients with no signs of anaemia, from patients with the anaemia associated with rheumatoid arthritis, with iron-deficiency anaemia and with pernicious anaemia are presented.

Results have been expressed as μg. of iron/100 mg. of protein.

METHODS

All apparatus was rendered free from iron by cleaning in 50% HCl and in water after the usual chromic acid treatment. The reagents were frequently checked for iron content.

Preparation of marrow samples

The aspirate, obtained by the technique described by Davidson (1941), was placed in a tube containing heparin as anticoagulant. Marrow particles were transferred by means of a pointed glass rod to a second tube containing 1 ml. of 0·9% (w/v) NaCl. After standing for about 1 hr. at 4° the particles were transferred to an all-glass homogenizer (Potter & Elvehjem, 1936) containing 1 ml. of water and ground to an even suspension. The homogenizer was rinsed with 0·5 ml. of water and this was added to the suspension. The quantity of tissue used was determined by the size of the sample, but 5–10 mg. wet wt. was found to be a convenient amount.

As the quantity of iron to be estimated was very small ($<1\,\mu$g.) a blank solution was prepared by the same procedure but with the marrow omitted.

Samples of both blank and marrow preparations were taken for the estimation of iron and of protein.

Iron estimation

The method was essentially that of Ramsay & Campbell (1954) suitably modified for the small amounts of tissue available.

Reagents. Sodium sulphite. 0·5M-soln. Dipyridyl reagent: 1% (w/v) $\alpha\alpha'$-dipyridyl in 10% (v/v) acetic acid. All reagents were saturated with CO before use.

Method. To 1 ml. of the suspension were added 0·5 ml. of Na_2SO_3 and 1·5 ml. of dipyridyl reagent. The solutions were mixed and heated in a boiling-water bath for 30 min., after which the volume was made up to 3 ml. with water. The solution was cooled and centrifuged at 4°. The intensity of the pink colour in the supernatant was measured against the blank in the Unicam SP. 600 spectrophotometer at 520 mμ.

Protein estimation

The method of Lowry, Rosebrough, Farr & Randall (1951) was used with suitable modification.

Reagents. Alkaline copper reagent: 1 ml. of 0·5%(w/v) $CuSO_4,5H_2O$ in 1% (w/v) sodium potassium tartrate added to 50 ml. of 2% (w/v) Na_2CO_3 (anhydrous) in 0·1N-NaOH. Folin and Ciocalteu reagent (British Drug Houses Ltd.) diluted to 0·1N-acid. Both reagents were prepared freshly before use.

Method. To 0·2 ml. of the suspension was added 2 ml. of the alkaline copper reagent and the mixture allowed to stand at room temperature for 10 min. A volume (1 ml.) of the Folin reagent was added rapidly with mixing and the solution again left at room temperature for 20 min. After centrifuging at 4° the blue colour in the supernatant was measured against the blank in the Unicam SP. 600 spectrophotometer at 750 mμ.

RESULTS

Reproducibility of the method. Several marrow samples were combined and six replicate estimations carried out. The average value for the iron content was found to be 0·45 μg. with a standard deviation of 0·08 μg., and that for the protein $18{\cdot}2 \pm 1{\cdot}4\,\mu$g.

Error of sampling. This was tested in two ways. Particles from an aspirate of marrow were divided into four parts and the iron content of each part was estimated. The average value was 85 μg./100 mg. of protein with a range of 81–90 μg. The iron content was determined in multiple aspirates of marrow from the sternum (three samples) and the iliac crests (two samples) obtained at necropsy from each of two individuals. The average value in the first patient was 42 μg. of iron/100 mg. of protein with a range of 31–53 μg. In the second patient the average was 219 μg. of iron with a range of 197–240 μg.

Comparison of results obtained by the quantitative and histological methods. A quantitative estimate of the iron content was made in the bone marrow from forty-nine individuals and sections of fixed tissue were stained by the Prussian blue method. For ease of comparison the results from the histological method have been grouped into those showing little or no detectable staining (group 1) and those showing definite staining (group 2). The iron content could then be compared in the two groups (Fig. 1). The mean value for group 1 was 45 μg. of iron/100 mg. of protein, with a standard deviation of 27 μg. and, for group 2, $72 \pm 35\,\mu$g. The difference between these values is highly significant ($P < 0{\cdot}01$).

Iron content of bone marrow. The iron content was estimated in marrow from sixteen patients in an Orthopaedic Ward, none of whom showed any signs of anaemia; from fifty-three patients suffering from the anaemia associated with rheumatoid arthritis; from six patients with iron-deficiency anaemia, and from three with pernicious anaemia. The results are shown in Table 1. Analysis of these results has shown that although the average iron content of the marrow in rheumatoid arthritis is reduced as compared with that of the non-anaemic control group, it does not reach the low level found

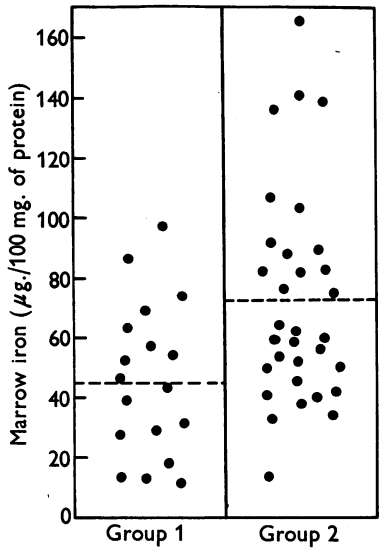

Fig. 1. A comparison of the values for the iron content of bone marrow as determined by the quantitative and Prussian blue methods. Group 1, little or no Prussian blue staining; group 2, definite Prussian blue staining.

Table 1. *Iron content of bone marrow in various anaemias*

Standard deviation and the number of observations (in parentheses) are also given.

Diagnosis	Iron content (μg./100 mg. of protein)
Control, non-anaemic	86±34 (16)
Rheumatoid arthritis	55±30 (53)
Iron-deficiency anaemia	12±5 (6)
Pernicious anaemia	86–136 (3)

in iron-deficiency anaemia. The difference between the average values in the two groups is highly significant ($P = 0{\cdot}01$).

DISCUSSION

Great advances have been made in the study of iron metabolism since the introduction of radioactive isotopes. From the studies of the interrelationship of ferritin and haemosiderin it would appear that both compounds are metabolically active. In view of this it is of importance, especially in bone marrow, to be able to measure the total non-haem iron when assessing the iron content of the tissue. Stress has been laid by Hutchison (1953) on the presence or absence of iron stainable by ferrocyanide in the diagnosis and treatment of anaemia. He claims that only in those patients whose marrow contains no haemosiderin will administration of iron increase haemopoiesis. Beutler, Drennan & Block (1954), however, have shown that a marked improvement in the haemoglobin level can occur following oral-iron therapy without the appearance of haemosiderin in the marrow. Only after prolonged administration of iron had increased the body stores of iron was haemosiderin detectable in the marrow. Thus although a negative Prussian blue test may indicate subnormal amounts of iron in the marrow, it gives no measure of the severity of the deficiency.

The method described above for the estimation of iron in small samples of bone marrow is simple and appears to be reliable. Replicate analysis of single and multiple samples has shown the accuracy to be sufficient for diagnostic purposes. It might be argued that protein is not a suitable constituent of the marrow on which to base the iron content and that deoxyribonucleic acid would give more reliable information in relation to the cellularity of the tissue. These considerations are outweighed by the accuracy of the method for the estimation of protein compared with that for phosphorus at the level encountered in this work. The use of the specific ultraviolet absorption for the estimation of both protein and deoxyribonucleic acid, as recently reported in a review of bone-marrow metabolism (Lajtha, 1957), might lead to an interesting comparison of results.

A good overall agreement between the quantitative and Prussian blue methods can be shown by dividing the results into two groups, those giving definite staining and those giving little or no staining. When individual results are considered, however, it will be seen from Fig. 1 that a marked disagreement may occur. It should be borne in mind that whereas the histological method is confined to the demonstration of iron in the form of haemosiderin, the quantitative method measures the total non-haem iron. Thus considerable disagreement may occur between individual results as estimated by the two methods.

The range of values obtained for the iron content of the marrow from non-anaemic subjects, from patients with pernicious anaemia and iron-deficiency anaemia is in agreement with that generally reported with the Prussian blue method (Rath & Finch, 1948; Davidson & Jennison, 1952). Richmond, Gardner, Roy & Duthie (1956) have studied the iron content of bone marrow from patients suffering from the anaemia associated with rheumatoid arthritis. Although no other sign of iron deficiency was found, 33% of the marrows examined contained no iron detectable by the Prussian blue test. The quantitative method now described was used in a small proportion of these cases, but no normal material was then available for comparison. The results now reported confirm the earlier findings that there is significantly less iron in the marrow in rheumatoid arthritis than is

normally present, but it has also been possible to show that only a minor degree of iron deficiency occurs and only in the rare case does the marrow-iron level reach the low values found in iron-deficiency anaemia. This method may therefore be of use in differentiating between degrees of iron deficiency.

SUMMARY

1. A simple method for the determination of the non-haem iron content of bone marrow is described which is of sufficient accuracy for diagnostic purposes.

2. Results obtained by this method have been compared with those obtained by the Prussian-blue staining method.

3. The non-haem iron content of bone marrow from both non-anaemic and anaemic subjects has been determined.

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The Lipids of Ram Spermatozoa

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Since the pioneering studies of Miescher (1878,1897) it has been known that spermatozoa contain a considerable amount of intracellular lipid. Miescher believed the lipid material, which he extracted from salmon spermatozoa, to consist of 50% lecithin, 35% neutral fat and 14% cholesterol. Studies by Mathews (1897) and Sano (1922) on the semen of various fishes also indicated the presence of a large amount of lecithin in the sperm cells. The lipids of sea-urchin sperm have been analysed by Cardin & Meara (1953) and found to consist of 26·0% phospholipids, 32·9% free fatty acids, 13·6% neutral fat, 9·2% sterols and 18·3% of other unsaponifiable material. The lipid content of bull sperm was first determined by Kölliker (1856), who showed that ether-extractable material accounted for 12% of the dry weight of the sperm. Miescher (1878), who performed the first analysis of the lipid extracted from bull sperm, thought that about half of it consisted of lecithin. More recently, determinations of total lipid and phospholipids in bull sperm were carried out by Lardy & Phillips (1941) and Zittle & O'Dell (1941). The latter authors found that 13% of the dry weight of bull sperm was lipid, the sperm heads containing 7% lipid, the middle pieces 6% and the tails 23%.

The suggestion that lipids occur in spermatozoa in a bound state, presumably in the form of lipoproteins, was made by Halpern (1945) on the basis of his experiments with salmon semen. Mayer and